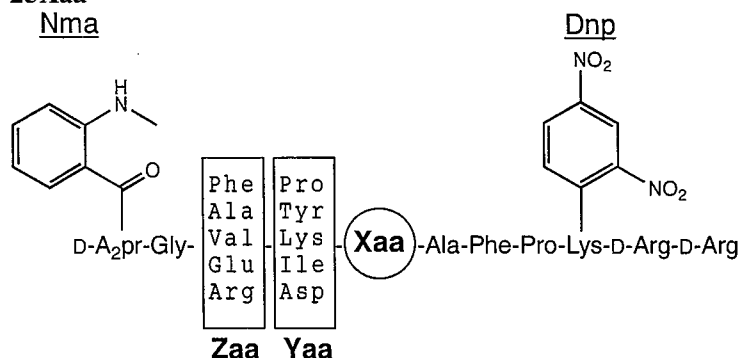# FRETS-25Xaa Series

* FRETS = Fluorescence Resonance Energy Transfer Substrates

Design of FRETS-25Xaa

Each substrate (#3701-v - #3719-v) in the FRETS-25Xaa series contains a highly fluorescent 2-(N-methylamino)benzoyl (Nma) group linked to the side chain of the amino-terminal D-A2pr residue, which is efficiently quenched by a 2,4-dinitrophenyl (Dnp) group linked to the ε-amino function of Lys. Xaa represents a fixed position of each of the 19 natural amino acids excluding Cys (*noted in product name #3701-v - #3719-v*). A mixture of 5 amino acid residues (P, Y, K, I, and D) is at the Yaa position along with a mixture of 5 amino acid residues (F, A, V, E, and R) at the Zaa position for each fixed Xaa. This provides a peptide mixture of 25 combinations of each Xaa series resulting in a combinatorial library totaling 475 peptide substrates. Both Nma and Dnp groups are linked to the side chain of the individual residues, allowing for the determination of the cleavage site by a specific enzyme through mass spectrometric analysis and Edman degradation as well.

Principle
When an enzyme of interest cleaves any peptide bond between D-A2pr(Nma) and Lys(Dnp) in the substrate, the fluorescence at λex = 340 nm and λem = 440 nm increases in proportion to the release of the Nma fluorophore from the internal Dnp quencher.

Reagents
1) Each substrate stock solutions: each FRETS-25Xaa (#3701-v - #3719-v) in 1.0 ml of DMSO (1 mM, total of peptides)
2) Reference compounds stock solution: a 1:1 mixture of two solutions of #3720-v and #3721-v, each of which is reconstituted by dissolving peptides in 0.5 ml of DMSO at the concentration of 2 mM (1 mM, each reference compound)
3) Enzyme solution: an enzyme of interest in an appropriate buffer
4) Buffer

Procedure for the deduction of the substrate specificity of an enzyme with unidentified cleavage specificity
Choose the proper conditions for the measurement, such as substrate concentration and sensitivity setting, depending on the purpose of the experiment and the instrument available. Described here is one of the recommended procedures for determining the enzymatic cleavage site by the combination of the fluorometric analysis and liquid chromatography-mass spectrometry (LC-MS) analysis.

i) Primary screening: selection of the favored Xaa
·Substrate solution for primary screening (PS solution): Dilute 20 µl of each of the above substrate stock solution with 1980 µl of an appropriate buffer (10 µM)
·Reference compounds solution for primary screening (PR solution): Dilute 20 µl of the above reference compounds stock solution with 1980 µl of an appropriate buffer (10 µM)

1) Set a fluorescence spectrophotometer at λex = 340 nm and λem = 440 nm
2) Mix one of the PS solution and the PR solution in ratios of 10/0, 9/1, 8/2, 5/5 and 0/10

3) Measure the fluorescence of the prepared solutions to obtain the calibration curve for the cleaved products
4) Pipette 200 μl each of all PS solutions into the cells and incubate them in the fluorescence spectrophotometer for 3 min (temperature equilibration)
5) Measure the fluorescence of each solution (initial fluorescence blank)
6) Add an appropriate volume of enzyme solution
7) Record the increase of the fluorescence intensity
8) Terminate the enzymatic reaction by using a proper inhibitor (leupeptin, E-64, pepstatin, EDTA and so on) or changing the pH of the reaction medium (using TCA, AcOH, NaOH and so on)
9) Choose the best Xaa-containing substrate for secondary screening

ii) Secondary screening: identification of the specificity of the enzyme (I)
·Substrate solution for secondary screening (SS solution): Dilute 200 μl of the stock solution of the best Xaa-containing substrate chosen by the above primary screening with 1800 μl of an appropriate buffer (100 μM)
·Reference compounds solution for secondary screening (SR solution): Dilute 200 μl of the above reference compounds stock solution with 1800 μl of an appropriate buffer (100 μM)

1) Set a fluorescence spectrophotometer at λex = 340 nm and λem = 440 nm
2) Mix the SS solution and the SR solution in ratios of 100/0, 95/5, 90/10, 80/20, 50/50 and 0/100
3) Measure the fluorescence of the prepared solutions to obtain the calibration curve for the cleaved products
4) Pipette 200 μl of the SS solution into the cells and incubate them in the fluorescence spectrophotometer for 3 min (temperature equilibration)
5) Measure the fluorescence of each solution (initial fluorescence blank)
6) Add an appropriate volume of enzyme solution
7) Record the increase of the fluorescence intensity
8) Terminate the enzymatic reaction by using a proper inhibitor or changing the pH of the reaction medium upon completion of the reaction at the points of 0%, 5%, 10% and 20% of the total
9) Subject 100 μl aliquots to LC-MS

iii) LC-MS: identification of the specificity of the enzyme (II)
·Analytical conditions
    column: ODS
    eluant: A) H_2O containing 0.05% TFA, B) CH_3CN containing 0.05% TFA
    gradient: 10% to 40% B) in A) over 50 min
    detection: UV at 220 nm and 400 nm or fluorescence

1) Inject 100 μl aliquots of each terminated solution at different stage of the reaction
2) Measure the MW of the cleaved product(s) in the peak(s) with the absorbance at 220 nm but not with 400 nm [identification of the N-terminal segment(s)]
3) Deduce their structure from the attached list of the theoretical MW for the cleaved products

* Comment 1: If the N-terminal segment has the identical retention time to the C-terminal segment or one of the starting uncleaved substrates, detection of the products by fluorescence is recommended.
* Comment 2: In the accidental case where the two products with the same MW (ex. Zaa-Yaa=Phe-Asp and Val-Tyr, Glu-Asp and Phe-Pro) are generated from one of the substrate, their analyses should be carried out by MS-MS sequencing and/or by Edman degradation.

Usefulness and limitation of FRETS-25Xaa series for screening of substrate specificities of proteases
We have confirmed that FRETS-25Xaa series are effectively used for the assay of numerous proteases such as trypsin, chymotyrpsin, elastase, thrombin, papain, calpain, pepsin and thermolysin. However, they did not work well for the assay of caspase-3 and furin, probably because they have only three changeable sites (Zaa-Yaa-Xaa) in each substrate (deficiency of P4 site). This fact implies that FRETS-25Xaa might not be applicable to the assay of an enzyme with wide range interacting sites with substrate.

FRETS-25Ser	Average	Monoisotopic	FRETS-25Ser	Average	Monoisotopic	FRETS-25Ser	Average	Monoisotopic	FRETS-25Ser	Average	Monoisotopic
A2pr(Nma)G	294.31	294.1328	A2pr(Nma)GVIS	593.67	593.3173	A2pr(Nma)GEDSA	696.66	696.2715	A2pr(Nma)GAPSAFP	864.94	864.4130
A2pr(Nma)GA	365.38	365.1699	A2pr(Nma)GVDS	595.60	595.2602	A2pr(Nma)GFPSA	696.75	696.3231	FPK(Dnp)rr	868.94	868.4304
A2pr(Nma)GV	393.44	393.2012	A2pr(Nma)GFY	604.65	604.2645	A2pr(Nma)GRYS	700.74	700.3293	A2pr(Nma)GRISAF	868.98	868.4555
A2pr(Nma)GE	423.42	423.1754	A2pr(Nma)GEPS	607.61	607.2602	A2pr(Nma)GRPSA	705.76	705.3558	A2pr(Nma)GRDSAF	870.91	870.3984
A2pr(Nma)GF	441.48	441.2012	A2pr(Nma)GVKS	608.69	608.3282	A2pr(Nma)GEKSA	709.75	709.3395	A2pr(Nma)GFKSAF	874.98	874.4337
A2pr(Nma)GR	450.49	450.2339	A2pr(Nma)GRY	613.67	613.2972	A2pr(Nma)GFISA	712.79	712.3544	A2pr(Nma)GAISAFP	880.99	880.4443
A2pr(Nma)GAP	462.50	462.2227	A2pr(Nma)GAYS	615.63	615.2653	A2pr(Nma)GFDSA	714.72	714.2973	A2pr(Nma)GADSAFP	882.92	882.3872
A2pr(Nma)GAI	478.54	478.2540	A2pr(Nma)GAPSA	620.65	620.2918	A2pr(Nma)GVYSA	714.77	714.3337	A2pr(Nma)GRKSAF	883.99	883.4664
A2pr(Nma)GAD	480.47	480.1969	A2pr(Nma)GEIS	623.66	623.2915	PK(Dnp)rr	721.77	721.3620	A2pr(Nma)GEYSAF	891.92	891.3763
A2pr(Nma)GVP	490.55	490.2540	K(Dnp)rr	624.65	624.3092	A2pr(Nma)GRISA	721.80	721.3871	A2pr(Nma)GVPSAFP	893.00	892.4443
A2pr(Nma)GAK	493.56	493.2649	A2pr(Nma)GEDS	625.59	625.2344	A2pr(Nma)GRDSA	723.73	723.3300	A2pr(Nma)GAKSAFP	896.00	895.4552
A2pr(Nma)GVI	506.60	506.2853	A2pr(Nma)GFPS	625.67	625.2860	A2pr(Nma)GFKSA	727.81	727.3653	A2pr(Nma)GVISAFP	909.04	908.4756
A2pr(Nma)GVD	508.52	508.2282	A2pr(Nma)GRPS	634.68	634.3187	A2pr(Nma)GRKSA	736.82	736.3980	A2pr(Nma)GFYSAF	909.98	909.4021
A2pr(Nma)GEP	520.54	520.2282	A2pr(Nma)GAISA	636.70	636.3231	A2pr(Nma)GEYSA	744.75	744.3079	A2pr(Nma)GVDSAFP	910.97	910.4185
A2pr(Nma)GVK	521.61	521.2962	A2pr(Nma)GADSA	638.63	638.2660	A2pr(Nma)GFYSA	762.81	762.3337	A2pr(Nma)GRYSAF	918.99	918.4348
A2pr(Nma)GAY	528.56	528.2332	A2pr(Nma)GEKS	638.67	638.3024	A2pr(Nma)GAPSAF	767.83	767.3602	A2pr(Nma)GEPSAFP	922.98	922.4185
A2pr(Nma)GEI	536.58	536.2595	A2pr(Nma)GFIS	641.72	641.3173	A2pr(Nma)GRYSA	771.82	771.3664	A2pr(Nma)GVKSAFP	924.05	923.4865
A2pr(Nma)GED	538.51	538.2023	A2pr(Nma)GFDS	643.64	643.2602	A2pr(Nma)GAISAF	783.87	783.3915	A2pr(Nma)GAYSAFP	931.00	930.4236
A2pr(Nma)GFP	538.60	538.2540	A2pr(Nma)GVYS	643.69	643.2966	A2pr(Nma)GADSAF	785.80	785.3344	A2pr(Nma)GEISAFP	939.02	938.4498
A2pr(Nma)GRP	547.61	547.2867	A2pr(Nma)GVPSA	648.71	648.3231	A2pr(Nma)GVPSAF	795.88	795.3915	AFPK(Dnp)rr	940.02	939.4675
A2pr(Nma)GAPS	549.58	549.2547	A2pr(Nma)GRIS	650.73	650.3500	A2pr(Nma)GAKSAF	798.89	798.4024	A2pr(Nma)GEDSAFP	940.95	940.3927
A2pr(Nma)GEK	551.59	551.2704	A2pr(Nma)GAKSA	651.71	651.3340	A2pr(Nma)GVISAF	811.92	811.4228	A2pr(Nma)GFPSAFP	941.04	940.4443
A2pr(Nma)GFI	554.64	554.2853	A2pr(Nma)GRDS	652.66	652.2929	A2pr(Nma)GVDSAF	813.85	813.3657	A2pr(Nma)GRPSAFP	950.05	949.4770
A2pr(Nma)GFD	556.57	556.2282	A2pr(Nma)GFKS	656.73	656.3282	A2pr(Nma)GEPSAF	825.86	825.3657	A2pr(Nma)GEKSAFP	954.04	953.4607
A2pr(Nma)GVY	556.61	556.2645	A2pr(Nma)GVISA	664.75	664.3544	A2pr(Nma)GVKSAF	826.94	826.4337	A2pr(Nma)GFISAFP	957.08	956.4756
A2pr(Nma)GRI	563.65	563.3180	A2pr(Nma)GRKS	665.74	665.3609	A2pr(Nma)GAYSAF	833.89	833.3708	A2pr(Nma)GFDSAFP	959.01	958.4185
A2pr(Nma)GRD	565.58	565.2609	A2pr(Nma)GVDSA	666.68	666.2973	A2pr(Nma)GEISAF	841.91	841.3970	A2pr(Nma)GVYSAFP	959.05	958.4549
A2pr(Nma)GAIS	565.62	565.2860	Ac-K(Dnp)rr	666.69	666.3198	A2pr(Nma)GEDSAF	843.84	843.3399	A2pr(Nma)GRISAFP	966.09	965.5083
A2pr(Nma)GADS	567.55	567.2289	A2pr(Nma)GEYS	673.67	673.2708	A2pr(Nma)GFPSAF	843.92	843.3915	A2pr(Nma)GRDSAFP	968.02	967.4512
A2pr(Nma)GFK	569.65	569.2962	A2pr(Nma)GEPSA	678.69	678.2973	A2pr(Nma)GRPSAF	852.94	852.4242	A2pr(Nma)GFKSAFP	972.10	971.4865
A2pr(Nma)GVPS	577.63	577.2860	A2pr(Nma)GVKSA	679.76	679.3653	A2pr(Nma)GEKSAF	856.92	856.4079	A2pr(Nma)GRKSAFP	981.11	980.5192
A2pr(Nma)GRK	578.66	578.3289	A2pr(Nma)GAYSA	686.71	686.3024	A2pr(Nma)GFISAF	859.97	859.4228	A2pr(Nma)GEYSAFP	989.04	988.4290
A2pr(Nma)GAKS	580.63	580.2969	A2pr(Nma)GFYS	691.73	691.2966	A2pr(Nma)GFDSAF	861.90	861.3657	A2pr(Nma)GFYSAFP	1007.10	1006.4549
A2pr(Nma)GEY	586.59	586.2387	A2pr(Nma)GEISA	694.73	694.3286	A2pr(Nma)GVYSAF	861.94	861.4021	A2pr(Nma)GRYSAFP	1016.11	1015.4876

FRETS-25Ser Component-masses

FRETS-25Ser	Average	Monoisotopic	FRETS-25Ser	Average	Monoisotopic	FRETS-25Ser	Average	Monoisotopic	FRETS-25Ser	Average	Monoisotopic
SAFPK(Dnp)rr	1027.09	1026.4995	A2pr(Nma)GRISAFPK(Dnp)	1260.36	1259.6047	A2pr(Nma)GAISAFPK(Dnp)r	1331.44	1330.6418	A2pr(Nma)GRKSAFPK(Dnp)r	1431.56	1430.7167
PSAFPK(Dnp)rr	1124.21	1123.5523	AYSAFPK(Dnp)rr	1261.35	1260.6000	A2pr(Nma)GADSAFPK(Dnp)r	1333.37	1332.5847	A2pr(Nma)GEYSAFPK(Dnp)r	1439.49	1438.6266
ISAFPK(Dnp)rr	1140.25	1139.5836	A2pr(Nma)GRDSAFPK(Dnp)	1262.29	1261.5476	FYSAFPK(Dnp)rr	1337.44	1336.6313	A2pr(Nma)GFYSAFPK(Dnp)r	1457.55	1456.6524
DSAFPK(Dnp)rr	1142.18	1141.5265	A2pr(Nma)GFKSAFPK(Dnp)	1266.36	1265.5829	GRPSAFPK(Dnp)rr	1337.45	1336.6749	A2pr(Nma)GRYSAFPK(Dnp)r	1466.56	1465.6851
KSAFPK(Dnp)rr	1155.27	1154.5945	GAISAFPK(Dnp)rr	1268.38	1267.6422	GEKSAFPK(Dnp)rr	1341.43	1340.6585	A2pr(Nma)GAPSAFPK(Dnp)rr	1471.58	1470.7116
A2pr(Nma)GAPSAFPK(Dnp)	1159.21	1158.5094	EISAFPK(Dnp)rr	1269.37	1268.6262	A2pr(Nma)GVPSAFPK(Dnp)r	1343.45	1342.6418	A2pr(Nma)GAISAFPK(Dnp)rr	1487.62	1486.7429
A2pr(Nma)GAISAFPK(Dnp)	1175.25	1174.5407	GADSAFPK(Dnp)rr	1270.31	1269.5850	GFISAFPK(Dnp)rr	1344.48	1343.6735	A2pr(Nma)GADSAFPK(Dnp)rr	1489.55	1488.6858
A2pr(Nma)GADSAFPK(Dnp)	1177.18	1176.4836	EDSAFPK(Dnp)rr	1271.30	1270.5691	GFDSAFPK(Dnp)rr	1346.41	1345.6163	A2pr(Nma)GVPSAFPK(Dnp)rr	1499.63	1498.7429
A2pr(Nma)GVPSAFPK(Dnp)	1187.26	1186.5407	FPSAFPK(Dnp)rr	1271.38	1270.6207	A2pr(Nma)GAKSAFPK(Dnp)r	1346.45	1345.6527	A2pr(Nma)GAKSAFPK(Dnp)rr	1502.64	1501.7538
A2pr(Nma)GAKSAFPK(Dnp)	1190.26	1189.5516	A2pr(Nma)GRKSAFPK(Dnp)	1275.37	1274.6156	GVYSAFPK(Dnp)rr	1346.45	1345.6527	A2pr(Nma)GVISAFPK(Dnp)rr	1515.67	1514.7742
YSAFPK(Dnp)rr	1190.27	1189.5629	GVPSAFPK(Dnp)rr	1280.39	1279.6422	RYSAFPK(Dnp)rr	1346.45	1345.6640	A2pr(Nma)GVDSAFPK(Dnp)rr	1517.60	1516.7171
APSAFPK(Dnp)rr	1195.29	1194.5894	RPSAFPK(Dnp)rr	1280.40	1279.6534	GRISAFPK(Dnp)rr	1353.49	1352.7062	A2pr(Nma)GEPSAFPK(Dnp)rr	1529.61	1528.7171
A2pr(Nma)GVISAFPK(Dnp)	1203.30	1202.5720	A2pr(Nma)GEYSAFPK(Dnp)	1283.30	1282.5255	GRDSAFPK(Dnp)rr	1355.42	1354.6490	A2pr(Nma)GVKSAFPK(Dnp)rr	1530.69	1529.7851
A2pr(Nma)GVDSAFPK(Dnp)	1205.23	1204.5149	GAKSAFPK(Dnp)rr	1283.40	1282.6531	A2pr(Nma)GVISAFPK(Dnp)r	1359.49	1358.6731	A2pr(Nma)GAYSAFPK(Dnp)rr	1537.64	1536.7222
AISAFPK(Dnp)rr	1211.33	1210.6207	EKSAFPK(Dnp)rr	1284.38	1283.6371	GFKSAFPK(Dnp)rr	1359.49	1358.6844	A2pr(Nma)GEISAFPK(Dnp)rr	1545.66	1544.7484
ADSAFPK(Dnp)rr	1213.26	1212.5636	FISAFPK(Dnp)rr	1287.43	1286.6520	A2pr(Nma)GVDSAFPK(Dnp)r	1361.42	1360.6160	A2pr(Nma)GEDSAFPK(Dnp)rr	1547.59	1546.6913
A2pr(Nma)GEPSAFPK(Dnp)	1217.24	1216.5149	FDSAFPK(Dnp)rr	1289.36	1288.5949	GRKSAFPK(Dnp)rr	1368.50	1367.7171	A2pr(Nma)GFPSAFPK(Dnp)rr	1547.67	1546.7429
A2pr(Nma)GVKSAFPK(Dnp)	1218.32	1217.5829	VYSAFPK(Dnp)rr	1289.40	1288.6313	A2pr(Nma)GEPSAFPK(Dnp)r	1373.43	1372.6160	A2pr(Nma)GRPSAFPK(Dnp)rr	1556.69	1555.7756
VPSAFPK(Dnp)rr	1223.34	1222.6207	GVISAFPK(Dnp)rr	1296.43	1295.6735	A2pr(Nma)GVKSAFPK(Dnp)r	1374.50	1373.6840	A2pr(Nma)GEKSAFPK(Dnp)rr	1560.67	1559.7593
A2pr(Nma)GAYSAFPK(Dnp)	1225.27	1224.5200	RISAFPK(Dnp)rr	1296.44	1295.6847	GEYSAFPK(Dnp)rr	1376.43	1375.6269	A2pr(Nma)GFISAFPK(Dnp)rr	1563.72	1562.7742
AKSAFPK(Dnp)rr	1226.34	1225.6316	GVDSAFPK(Dnp)rr	1298.36	1297.6163	A2pr(Nma)GAYSAFPK(Dnp)r	1381.45	1380.6211	A2pr(Nma)GFDSAFPK(Dnp)rr	1565.65	1564.7171
A2pr(Nma)GEISAFPK(Dnp)	1233.29	1232.5462	RDSAFPK(Dnp)rr	1298.37	1297.6276	A2pr(Nma)GEISAFPK(Dnp)r	1389.47	1388.6473	A2pr(Nma)GVYSAFPK(Dnp)rr	1565.69	1564.7535
A2pr(Nma)GEDSAFPK(Dnp)	1235.22	1234.4891	A2pr(Nma)GFYSAFPK(Dnp)	1301.36	1300.5513	A2pr(Nma)GEDSAFPK(Dnp)r	1391.40	1390.5902	A2pr(Nma)GRISAFPK(Dnp)rr	1572.73	1571.8069
A2pr(Nma)GFPSAFPK(Dnp)	1235.30	1234.5407	FKSAFPK(Dnp)rr	1302.44	1301.6629	A2pr(Nma)GFPSAFPK(Dnp)r	1391.49	1390.6418	A2pr(Nma)GRDSAFPK(Dnp)rr	1574.66	1573.7498
VISAFPK(Dnp)rr	1239.38	1238.6520	A2pr(Nma)GRYSAFPK(Dnp)	1310.37	1309.5840	GFYSAFPK(Dnp)rr	1394.49	1393.6527	A2pr(Nma)GFKSAFPK(Dnp)rr	1578.73	1577.7851
VDSAFPK(Dnp)rr	1241.31	1240.5949	GEPSAFPK(Dnp)rr	1310.37	1309.6163	A2pr(Nma)GRPSAFPK(Dnp)r	1400.50	1399.6745	A2pr(Nma)GRKSAFPK(Dnp)rr	1587.74	1586.8178
A2pr(Nma)GRPSAFPK(Dnp)	1244.31	1243.5734	GVKSAFPK(Dnp)rr	1311.45	1310.6844	GRYSAFPK(Dnp)rr	1403.50	1402.6854	A2pr(Nma)GEYSAFPK(Dnp)rr	1595.67	1594.7277
A2pr(Nma)GEKSAFPK(Dnp)	1248.30	1247.5571	RKSAFPK(Dnp)rr	1311.45	1310.6956	A2pr(Nma)GEKSAFPK(Dnp)r	1404.49	1403.6582	A2pr(Nma)GFYSAFPK(Dnp)rr	1613.73	1612.7535
A2pr(Nma)GFISAFPK(Dnp)	1251.35	1250.5720	A2pr(Nma)GAPSAFPK(Dnp)r	1315.39	1314.6105	A2pr(Nma)GFISAFPK(Dnp)r	1407.53	1406.6731	A2pr(Nma)GRYSAFPK(Dnp)rr	1622.74	1621.7862
GAPSAFPK(Dnp)rr	1252.34	1251.6109	GAYSAFPK(Dnp)rr	1318.40	1317.6214	A2pr(Nma)GFDSAFPK(Dnp)r	1409.46	1408.6160			
A2pr(Nma)GFDSAFPK(Dnp)	1253.28	1252.5149	EYSAFPK(Dnp)rr	1319.38	1318.6054	A2pr(Nma)GVYSAFPK(Dnp)r	1409.50	1408.6524			
A2pr(Nma)GVYSAFPK(Dnp)	1253.32	1252.5513	GEISAFPK(Dnp)rr	1326.42	1325.6476	A2pr(Nma)GRISAFPK(Dnp)r	1416.54	1415.7058			
EPSAFPK(Dnp)rr	1253.32	1252.5949	GEDSAFPK(Dnp)rr	1328.35	1327.5905	A2pr(Nma)GRDSAFPK(Dnp)r	1418.47	1417.6487			
VKSAFPK(Dnp)rr	1254.40	1253.6629	GFPSAFPK(Dnp)rr	1328.43	1327.6422	A2pr(Nma)GFKSAFPK(Dnp)r	1422.55	1421.6840			